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^{32}P-Postlabeling Assay for Carcinogen-DNA Adducts: Nuclease P_1-Mediated Enhancement of Its Sensitivity and Applications

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Exceedingly sensitive assays are required for the detection of DNA adducts formed in humans exposed to low levels of environmental genotoxicants and therapeutic drugs. A ^{32}P-postlabeling procedure for detection and quantitation of aromatic carcinogen-DNA lesions with a sensitivity limit of 1 adduct in 10^7 to 10^8 nucleotides has been described previously. In the standard procedure, DNA is enzymatically digested to 3′-phosphorylated normal and adducted mononucleotides, which are ^{32}P-labeled at their 5′-hydroxyl groups by T4 polynucleotide kinase-catalyzed [^{32}P]phosphate transfer from [γ-^{32}P]ATP. ^{32}P-labeled derivatives are resolved by TLC, detected by autoradiography, and quantitated by counting. This assay has been recently utilized for the determination and partial characterization of DNA adducts formed in somatic and reproductive tissues of rats given the clinically used anticancer drug, mitomycin C. The drug exhibits similar levels of covalent binding to DNA in most tissues. Further studies have revealed that adducted nucleotides are primarily guanine derivatives that are resistant to 3′-dephosphorylation by *Penicillium citrinum* nuclease P_1. The latter observation has been utilized to enhance the ^{32}P-assay's sensitivity to 1 adduct in 10^{10} nucleotides for a 10-μg DNA sample by postincubation of DNA digests with nuclease P_1 before ^{32}P-labeling. The enzyme dephosphorylates the normal nucleotides but not most aromatic and bulky nonaromatic adducts, so that only the latter serve as substrates for the kinase-catalyzed labeling reaction. The new assay has also shown utility in the analysis of very low levels of age- and tissue-related DNA modifications, which might arise from dietary or endogenous compounds, in untreated rats and in humans.

Introduction

It is well documented that naturally occurring and synthetic chemicals play a role in the etiology of human cancer (*1*). In experimental animals and cultured human cells, the majority, but not all, of mutagenic and carcinogenic compounds are converted via chemical or metabolic activation to electrophiles; these reactive intermediates interact with DNA, RNA, and proteins, resulting in the formation of covalent adducts (*2,3*). Several lines of evidence suggest that DNA is the critical target molecule in the initiation of multistage chemical carcinogenesis. First, most carcinogens are mutagens, i.e., they react with DNA (*4*). Second, the carcinogenicity of a series of polycyclic aromatic hydrocarbons correlates with their binding to DNA, rather than to RNA and protein (*5*). Third, defects in DNA repair such as in xeroderma pigmentosum predispose to cancer development (*6*). Last, *in vitro* modification of the *ras* protooncogene with the ultimate carcinogens of benzo[a]pyrene or 2-acetylaminofluorene generates a transforming oncogene when introduced into NIH 3T3 cells (*7*). Methods for the detection of DNA alterations or damage are thus essential for the identification of potential carcinogens/mutagens in the human environment, and their application assists in the prevention or minimization of exposures to such compounds.

Several powerful short-term *in vitro* assays employing bacteria, lower eukaryotes, or mammalian cells in culture have been developed to detect genotoxic activity of chemicals (*8,9*). Since these systems are unable to convert carcinogens to electrophilic species, exogenous sources of metabolic enzymes are added. However, these *in vitro* assays do not completely mimic the *in vivo* situation, which entails complex biological interactions such as absorption, distribution, activation, detoxification, and repair of DNA damage. This makes it difficult to extrapolate results of *in vitro* tests to the situation in whole animals and humans. Therefore, a need is apparent for the development of *in vivo* assays, preferably assays that are capable of directly measuring DNA damage. Radioactive carcinogens or antibodies specific to known adducts have been utilized in such studies (*10,11*). Neither approach can be readily applied

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to the large number of chemicals present in the human environment; hence, alternative techniques have been sought. A general and sensitive approach for the measurement of DNA lesions formed with nonradioactive carcinogens has been described (*12–15*), in which normal and adducted nucleotides, generated by nuclease digests of DNA modified *in vivo* or *in vitro* with a compound of interest, are labeled with ^{32}P and detected and quantitated after TLC. The ^{32}P-approach has been applied to approximately 100 test chemicals, comprising arylamines and derivatives, azo compounds, nitroaromatics, polycyclic aromatic hydrocarbons, and methylating agents (*14*), as well as mycotoxins (*16*), heterocyclic polycyclic aromatics (*17*), alkenylbenzene derivatives (*18,19*), and estrogens (*20*). With each carcinogen tested, ^{32}P-labeled DNA adducts were readily detected, showing the potential use of the ^{32}P-assay as a short-term *in vivo* test for screening genotoxic chemicals. In this review, we describe results obtained by adapting the method to mitomycin C (MMC) (*21*), a clinically used anticancer drug produced by *Streptomyces caespitosus* (*22*). In addition, we discuss a minor modification of the standard procedure, which enhances the method's sensitivity about 1000-fold, i.e., to 1 adduct in approximately 10^{10} DNA nucleotides (*23*), and applications of this approach (*24–26*).

Materials and Methods

The sources of biochemical, chromatographic, and autoradiographic materials have been documented previously (*12–15*). [γ-^{32}P]ATP and polyethyleneimine (PEI)-cellulose sheets were prepared in the laboratory (*13,27*). For analysis of the tissue distribution of MMC-DNA adducts, Fischer 344 male and female rats (approximately 150 g) were given IP 9 mg/kg MMC in 3.6 mL of 0.9% NaCl; control rats received 0.9% NaCl. Tissues were collected 24 hr later, and DNA was isolated by a modified digestion and extraction procedure (*21*). DNA adducted with 7,12-dimethylbenz-[a]anthracene (DMBA) [relative adduct labeling (RAL) = 34.4×10^{-7}] was isolated from the skin of mice 24 hr after topical application of 1.2 μmole DMBA. BP-DNA (RAL = 10.8×10^{-7}] and ABP-DNA (RAL = 105.8×10^{-7}) were extracted from maternal intestine and maternal liver, respectively, 24 hr after *per os* administration of 200 μmole/kg benzo[a]pyrene (BP) or 800 μmole/kg 4-aminobiphenyl (ABP) to pregnant ICR mice on day 18 of gestation (*28*). DBC-DNA (RAL = 561.1×10^{-7}) was obtained from female BALB/c mouse liver 24 hr after topical application to the skin of 80 μmole/kg DBC (M. E. Schurdak and K. Randerath, in preparation).

^{32}P-postlabeling analysis of DNA adducts was performed as described previously (*12–15,21,23,29*). DNA was enzymatically digested to 3′-mononucleotides (dNp) (*30*). In the standard assay, 0.17 μg of DNA digest (50 μM dNp) was ^{32}P-labeled with 60 μM [γ-^{32}P]ATP (250–600 Ci/mmole) (*21,23*), and any unused [γ-^{32}P]ATP was converted to $^{32}P_i$ by digestion with apyrase. In the adduct intensification procedure, 2 μg of DNA digest (400 μM dNp) was ^{32}P-labeled with 1.7 μM carrier-free [γ-^{32}P]ATP (approximately 4000 Ci/mmole) (*21,29*). In the nuclease P_1-enhanced version of the assay, 10 μg of DNA digest was treated with nuclease P_1 (6 μg) for 40 min to remove normal nucleotides, then ^{32}P-labeled with excess carrier-free [γ-^{32}P]ATP (*23*). Purification and separation of ^{32}P-labeled adducted nucleotides was performed by PEI-cellulose anion-exchange TLC in the case of adducts from BP, DMBA, DBC, and 4-ABP (*21*), whereas for MMC adducts, a combination of octadecyl silane reversed-phase TLC and PEI-cellulose TLC was employed (*21*). After detection by screen-enhanced autoradiography, adduct spots were cut out for quantitation from replicate maps and counted by Cerenkov assay. Appropriate blank areas of the chromatograms were also assayed and their count rates subtracted from sample count rates. Adduct levels were calculated as relative adduct labeling (RAL), which is the ratio of count rates of adducted nucleotides to count rates of total (adduct plus normal) nucleotides. The latter were evaluated by TLC of an aliquot of the labeled solution (*21*). In the intensification procedure, apparent RAL values (<RAL>) were divided by adduct intensification factors to obtain actual RAL values (*21,29*). After nuclease P_1 treatment, which removes normal nucleotides prior to ^{32}P-labeling, the specific activity of [γ-^{32}P]ATP was determined by ^{32}P-labeling of dAp and utilized for RAL calculations (*23*). A value of RAL $\times 10^7$ corresponds to 1 adduct in 10^7 nucleotides or 0.3 pmole of adduct/mg DNA, provided that adducts are labeled and recovered quantitatively. Using DNA modified *in vivo* with [^{3}H]1′-hydroxy-2′,3′-dehydroestragole (*31*) or *in vitro* with [^{3}H]*N*-hydroxy-2-aminofluorene or [^{3}H]ben- zo[a]pyrene diol epoxide I (*13*), the levels of adducts determined by ^{32}P-postlabeling analysis have been shown to be 50 to 80% of those obtained by analysis of the tritiated adducts.

Results and Discussion

^{32}P-Analysis of MMC-DNA Adducts

MMC, a potent antitumor antibiotic, is a drug used clinically for the treatment of cancers of stomach, pancreas, lung, cervix, bladder, and breast (*22*). It is carcinogenic to rats and generates mutations in spermatogonia of male mice. The compound produces sister chromatid exchanges in bone marrow and testis of rats and mice and in lymphocytes of humans following *in vivo* treatments (*22,32*). In *in vitro*, chemically or enzymatically reduced MMC has been shown to react with DNA, forming monofunctional and bifunctional adducts in ratios, which vary depending on the reductive conditions (*33,34*); however, little information is available to date on *in vivo* adduction of tissue DNAs by MMC (*23*), probably because of unavailability of the drug in radiolabeled form. Using the ^{32}P-postlabeling assay (Fig. 1), we have studied the formation of MMC-DNA adducts in several somatic and reproductive tissues of

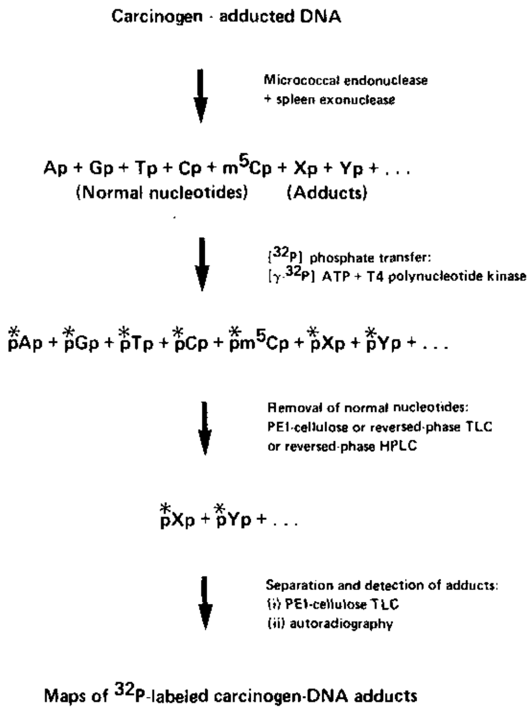

FIGURE 1. The basic features of ^{32}P-postlabeling assay for carcinogen-adducted DNA. The ^{32}P-assay involves four steps: digestion of DNA, ^{32}P-labeling of the digestion products, removal of ^{32}P-labeled nonadduct components, and TLC mapping of the [^{32}P]adducts. Asterisks indicate the position of the ^{32}P-label. Purification of adducted nucleotides derived from carcinogens having two or more aromatic rings (e.g., BP, DMBA, DBC, and ABP) is accomplished by PEI-cellulose TLC (*13–15*), while for adducts carrying a single aromatic ring (e.g., alkenylbenzenes and sterigmatocystin) or a bulky nonaromatic ring (e.g., MMC), octadecyl silane reversed-phase TLC is preferred (*16,18,19,21*).

rats at 24 hr after administration of 9 mg/kg of MMC. ^{32}P-fingerprints of DNA adducts in some selected tissues are shown in Figure 2. The map of bladder DNA showed 10 extra spots that were absent from the control DNA map (panel *a*). DNA adduct patterns were qualitatively similar in different tissues, except that in less modified DNA samples (e.g., from brain and testis), not all the adduct spots were detected. Since MMC-DNA digests were ^{32}P-labeled with [γ-^{32}P]ATP at a concentration below that of dNp to increase the sensitivity of the ^{32}P-assay (adduct-intensification procedure), ^{32}P-incorporation into adducts was corrected by intensification factors (*21*) for RAL calculations. Figure 3 shows RAL values of adduct 1 and of total ^{32}P-labeled MMC derivatives in DNA specimens isolated from 14 tissues of female rats (from left to right), as well as from liver and testis of male rats (the last two bars from the right) at 24 hr after IP administration of a 9 mg/kg dose of MMC. On average, adduct 1 comprised 71 ($\pm$ 5%) of the total. With the exception of brain, thymus, and spleen, total adduct levels varied within a two-fold range in 11 tissues in female rats, i.e., bladder, colon, esophagus, heart, kidney, liver, lung, ovary, pancreas, small intestine, and stomach (9.6–21.9 adducts/10^7 nucleotides). The ubiquitous binding of the drug to DNA in tissues is consistent with the carcinogenic (*35*) and the cytotoxic and necrogenic (*36*) effects of the drug in multiple organs of rats. The exceptionally low level of DNA modification in brain (0.7 adduct/10^7 nucleotides) was probably due to poor penetration by MMC of the blood-brain barrier. Liver DNA adduction was 32% lower in male compared with female rats. Ovarian DNA was 5.3 times more highly modified than was testicular DNA.

We have partially characterized the *in vivo* adducts by cochromatography with [^{32}P]adduct standards generated *in vitro* by the reaction of $NaBH_4$-reduced MMC with DNA and polydeoxyribonucleotides, followed by ^{32}P-labeling (*21*). With rat liver DNA modified *in vitro*, 10 [^{32}P]adduct spots were detected, which were chromatographically identical to those formed *in vivo*, demonstrating that activation of MMC occurred via reduction. *In vivo* adducts 1 to 5 were guanine derivatives, as they cochromatographed with adducts formed with poly dG. MMC-poly dC gave two adducts comigrating with *in vivo* adducts 7 and 9, while MMC-poly d(AT) gave two adducts cochromatographing with *in vivo* adducts 8 and 10.

While *in vivo* and *in vitro* MMC-DNAs showed two main adducts and eight minor derivatives by ^{32}P-analysis, formation of only one major adduct (> 90%) and two minor (2–5%) products has been reported with *in vitro*-modified DNA on the basis of HPLC and NMR analysis (*33,34*). The greater number of adducts seen by the ^{32}P-assay could be due to its higher sensitivity or incomplete digestion of DNA giving oligonucleotide adducts, such as those previously observed with sterigmatocystin-modified DNA (*16*). We have employed nuclease P_1 for the characterization of oligonucleotide adducts. This enzyme cleaves 3′-phosphomonester and 3′,5′-phosphodiester bonds of polynucleotides, yielding 5′-phosphorylated nucleotides. Following nuclease P_1 treatment, oligonucleotide adducts of the structure *pNpXp (*16*) should give *pN, pX, and P_i as the products, where * denotes the ^{32}P-label, N is normal nucleotide, X is a MMC adduct, and P_i is inorganic phosphate. The MMC-modified deoxyribonucleoside 3′,5′-bisphosphate, *pXp, on the other hand, should give *pX and P_i. When ^{32}P-labeled *in vivo* MMC adducts 1 to 4 were isolated and digested with nuclease P_1, neither *pN nor *pX were formed, while, as expected, reference normal nucleotides (*pNp) were hydrolyzed to *pN, showing that adducted nucleotides were resistant to 3′-dephosphorylation by this enzyme. We asked if this property of the enzyme could be utilized to enhance the sensitivity of the ^{32}P-assay further. As detailed in the next section, a 500- to 1000-fold increase in sensitivity of the procedure could be accomplished by including a nuclease P_1 treatment step prior to ^{32}P-labeling (*23*).

Nuclease P_1-Enhanced Version of the ^{32}P-Assay and Its Applications

This is a slight modification of the standard procedure, entailing a 40-min incubation of 3′-nucleotides in the

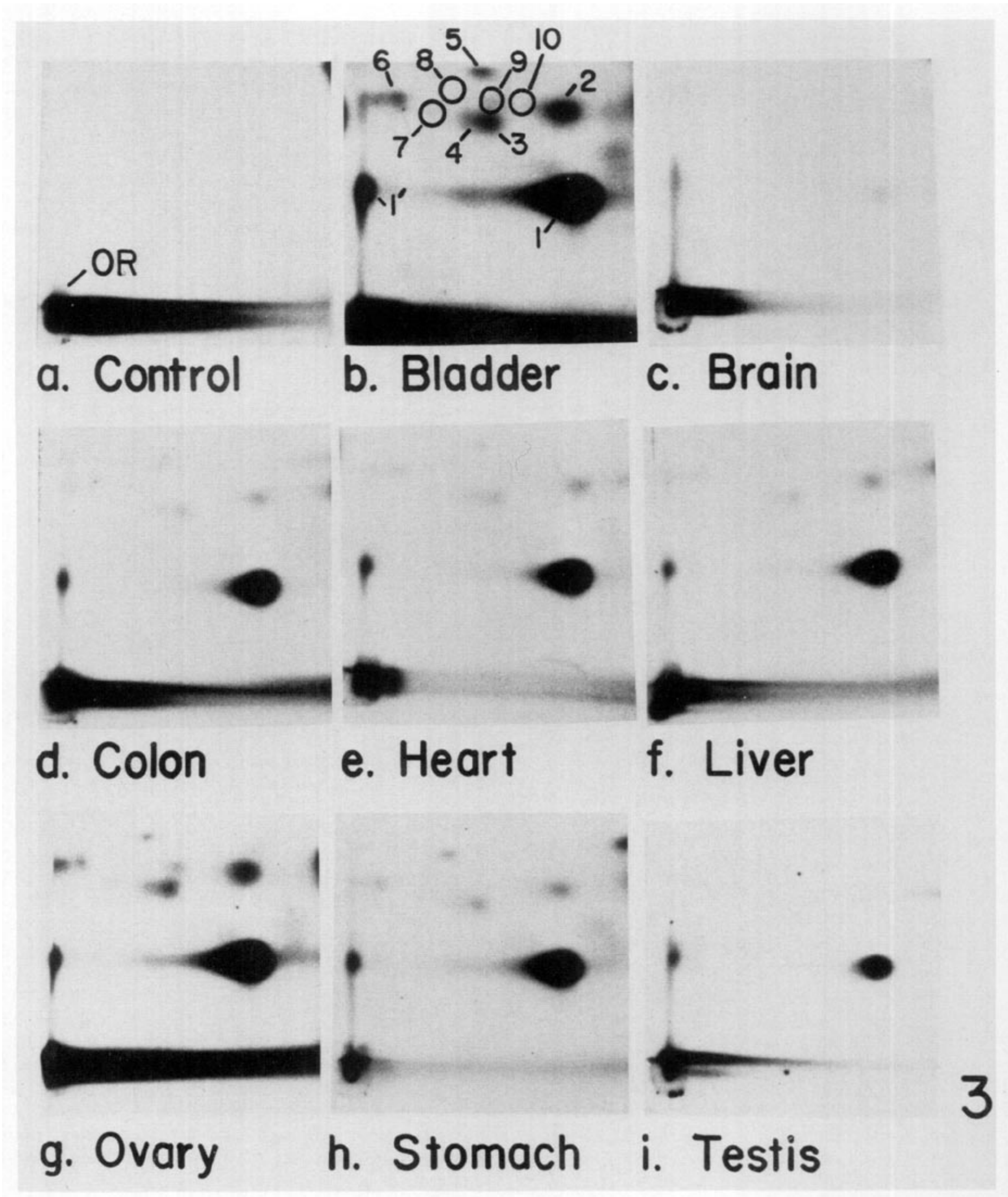

FIGURE 2. Autoradiograms of PEI-cellulose maps of ^{32}P-labeled MMC-DNA adducts. Samples were (*a*) bladder DNA from untreated rats; (*b–h*) DNAs from the indicated tissues of MMC-treated female rats; and (*i*) testicular DNA from male rats. Labeled DNA digests were prepared according to the scheme shown in Fig. 1 under adduct intensification conditions. Adducted [^{32}P]nucleotides were freed of normal [^{32}P]nucleotides by reversed-phase TLC at 4°C, contact-transferred and resolved by two-dimensional PEI-cellulose TLC (*21,23*), located by screen-intensified autoradiography at −80°C for 6 hr. Faint spots (nos. 7–10), requiring longer exposure times (1–2 days), have been circled. Spot 1′ was a fraction of adduct 1 retained during chromatographic development (*21*).

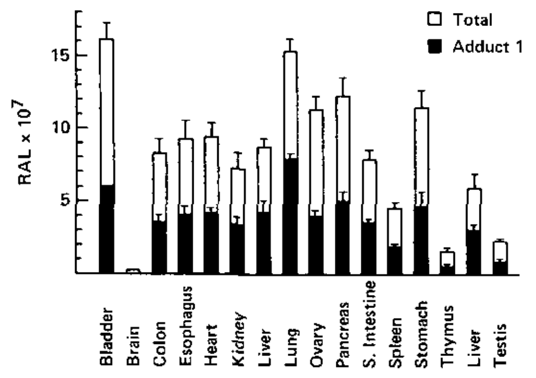

FIGURE 3. Levels of MMC adduct 1 and total adducts in different tissue DNAs of female and male rats given MMC. Adduct spots shown in Fig. 1 were cut from quadruplicate maps and counted. Binding is expressed as RAL values, as outlined in "Materials and Methods." Since the recovery of adduct 1 was 47.5%, multiplication of its RAL values by a factor of 2.11 yields actual RAL values (*21*). Bars indicate mean SD.

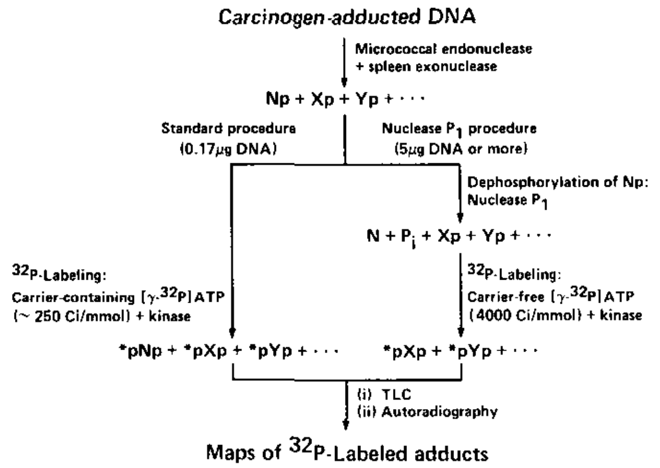

FIGURE 4. Experimental strategy employed in standard and nuclease P$_1$-enhanced procedures for ^{32}P-postlabeling analysis of carcinogen-DNA adducts. Nuclease P$_1$ dephosphorylates 3′-monophosphates of normal nucleotides, but not adducted nucleotides, so that the latter are enriched prior to ^{32}P-labeling. Np indicates normal nucleotides (dGp, dAp, dCp, dm^{5}Cp, and dTp). Xp, Yp denote adducted deoxyribonucleotides. See legend of Fig. 1 for additional details.

initial micrococcal endonuclease/spleen phosphodiesterase digests of DNA with nuclease P$_1$ (Fig. 4). Nuclease P$_1$ dephosphorylates normal, but not adducted, nucleotides to nucleosides. Since nucleosides are not substrates for polynucleotide kinase, only adducted nucleotides are ^{32}P-labeled in the subsequent labeling reaction. The enzymatic removal of normal nucleotides enables the specific labeling of adducts in up to 20 μg of DNA with excess [γ-^{32}P]ATP of high specific activity (approximately 4000 Ci/mmole). The assay's sensitivity is increased about 1000-fold compared with the standard procedure, in which normal and adducted nucleotides from 0.17 μg of DNA are simultaneously labeled with approximately 250 Ci/mmole of [γ-^{32}P]ATP. The enhancement is due to two factors: the labeling of adducts derived from a larger amount of DNA and the use of [γ-^{32}P]ATP of high specific activity. The nuclease P$_1$ procedure was validated with respect to its applicability to the measurement of DNA adducts formed with structurally diverse carcinogens. As examples, Figure 5 shows the ^{32}P-adduct patterns of BP-DNA, DMBA-DNA, DBC-DNA and ABP-DNA, respectively, analyzed by the nuclease P$_1$ procedure (panels *a–d*) and under standard conditions (panels *e–h*). Adduct patterns obtained by the two assays were qualitatively similar, but large quantitative differences were observed. This was indicated by a 50 to 250 times shorter film exposure for the upper row of autoradiograms and the more sensitive detection of weak spots. Counting of adduct fractions shown in Figure 5, as well as adducts of safrole and MMC (*23*), revealed that a total of 30 out of 34 adducts were enhanced 300- to 1000-fold in terms of ^{32}P-incorporation. Recoveries were 50 to 100% after nuclease P$_1$ treatment compared with the standard assay. These results showed that most aromatic and bulky nonaromatic adducted nucleotides were virtually resistant to 3′-dephosphorylation by nuclease P$_1$. Recoveries of two ABP adducts (Fig. 5, nos. 1 and 4) were lower (4–7%), but the gain in sensitivity was still 30- to 60-fold in relation to standard conditions. Two minor adducts, i.e., an ABP derivative (Fig. 5, no. 3) and a safrole derivative, showed properties similar to normal nucleotides upon nuclease P$_1$ treatment (*23*).

The nuclease P$_1$-enhanced version of the ^{32}P-assay has already shown utility in a number of cases requiring the analysis of very low DNA adduct levels. First, using this assay, we have detected the age- and tissue-dependent formation of covalent DNA modifications (termed I-compounds) in liver, kidney, lung, and heart of untreated Sprague-Dawley rats of different ages (*24*). This DNA modification could be due to environmental (e.g., dietary) factors or endogenous DNA-reactive metabolites (*20*) and may play a role in spontaneous tumor induction or aging (*24*). Second, putative aromatic DNA derivatives, which may be related to I-compounds seen in untreated rats, have also been observed in placenta (*26*), bone marrow (*25*), and peripheral blood leukocytes (*25*) of nonsmoking humans. Third, the new assay has recently been applied to DNA samples from placenta and white blood cells of nonsmoking, pregnant women who had a history of exposure to residential wood smoke, i.e., a mixture known to contain carcinogenic polycyclic aromatic hydrocarbons. No exposure-related adducts were detected, showing that residential wood smoke fails to induce levels of covalent DNA damage comparable to cigarette smoke (*26*). Last, the nuclease P$_1$ version of the ^{32}P-postlabeling assay is considerably more sensitive for many aromatic, including cigarette smoke-induced adducts, than the intensification version of the assay previously employed for the detection of adducts in smoker tissues [(*37*) and E. Randerath and K. Randerath, unpublished results].

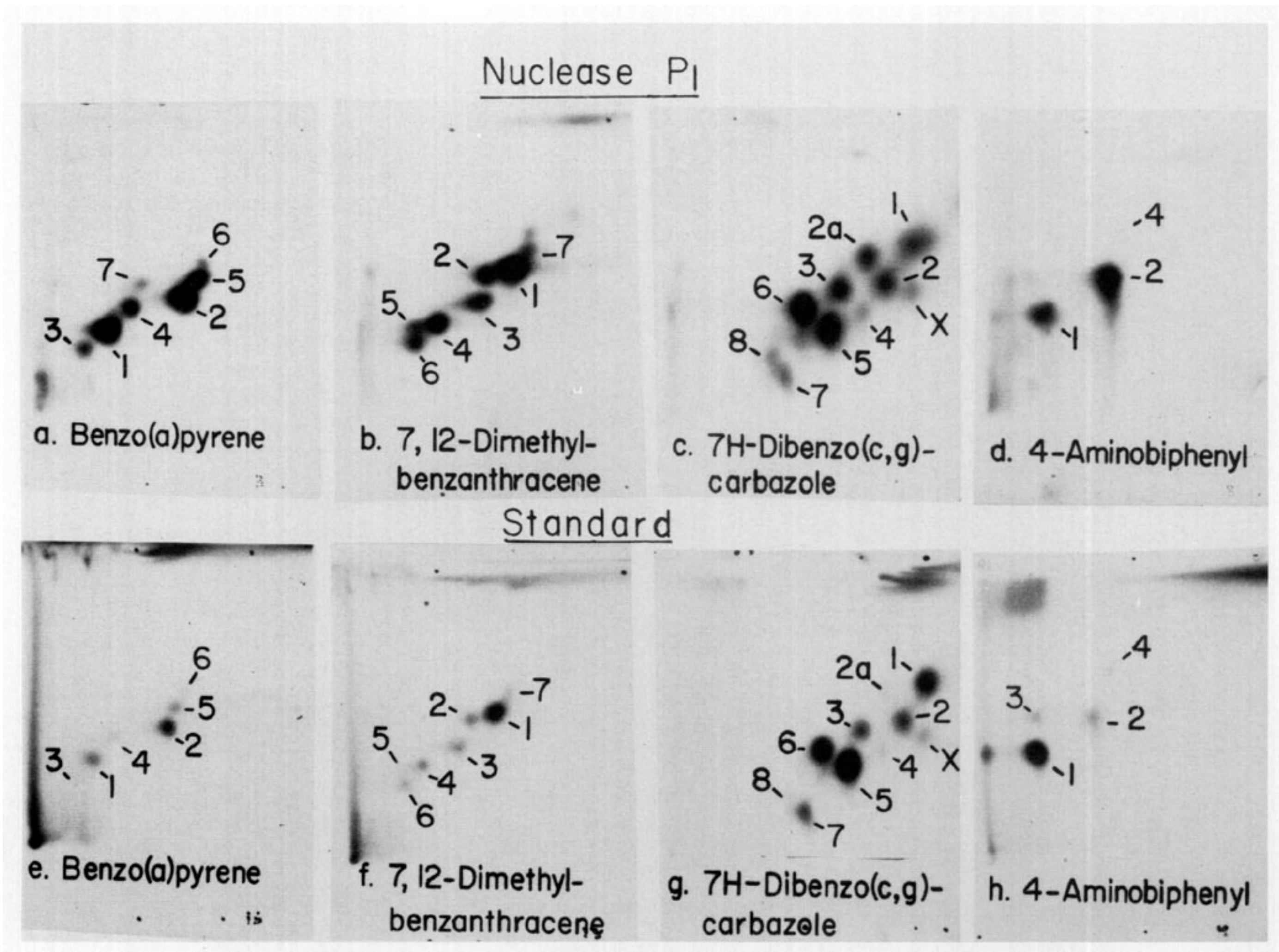

FIGURE 5. Comparison of nuclease P_1 and standard assays for BP-, DMBA-, DBC-, and ABP-DNA adducts. Autoradiograms of PEI-cellulose TLC maps of the indicated ^{32}P-labeled carcinogen-DNA adducts obtained with (*a–d*) and without (*e–h*) nuclease P_1 enhancement. DNAs were *in vivo* modified samples prepared as described in "Materials and Methods" (*23*). Labeled adducts in DNA digests were purified, contact-transferred, and resolved by two-dimensional PEI-cellulose TLC (*23*). The assignment of adduct numbers was identical to that adopted previously for [^{32}P]adducts of BP (*28*), DMBA (*29*), DBC (*17*), and ABP (*28*). Autoradiographic conditions employing Kodak XAR-5 films and Du Pont Lightning Plus screens were: (*a*) 23°C for 2.5 hr; (*b–d*) 23°C for 15 min; (*e*) −80°C for 36 hr; (*f*) −80°C for 3 hr. Note that the sensitivity of the X-ray film is increased approximately fourfold at −80°C relative to 23°C.

Conclusions

In vivo formation of carcinogen-DNA adducts in various somatic and reproductive tissues of experimental animals can be measured by a ^{32}P-postlabeling assay, as documented in this report with mitomycin C, a clinically used drug. The ^{32}P-assay is very sensitive, especially in combination with nuclease P_1 enhancement, which enables the detection and quantitation of most aromatic and bulky nonaromatic adducts at frequencies as low as 1 lesion per 10^{10} DNA nucleotides. In addition to its already demonstrated utility for the detection of DNA adducts in smokers, the new assay may potentially be applicable to DNA adduct measurements in experimental animals given single or mixed carcinogens at low dose levels that correspond to human exposures.

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